Kainate receptor-mediated synaptic transmissions in the adult rodent insular cortex

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Abstract

Kainate (KA) receptors are expressed widely in the central nervous systems, and regulate both excitatory and inhibitory synaptic transmission. KA receptors play important roles in fear memory, anxiety and pain. However, little is known about their function in synaptic transmission in the insular cortex (IC), a critical region for taste, memory and pain. Here we show that KA receptors contribute to fast synaptic transmission in neurons in all layers of the IC using whole cell patch-clamp recordings. In the presence of a GABA<sub>A</sub> receptor antagonist picrotoxin, a NMDA receptor antagonist AP-5 and a selective AMPA receptor antagonist GYKI 53655, KA receptor-mediated excitatory postsynaptic currents (KA EPSCs) was revealed. We found KA EPSCs are about 5-10% of AMPA/KA EPSCs in all layers of the adult mouse IC. Similar results were found in adult rat IC. KA-EPSCs had a significantly slower rise time course and decay time constant compared with AMPA receptor-mediated EPSCs. High frequency repetitive stimulations at 200 Hz significantly facilitated the summation of KA EPSCs. Additionally, genetic deletion of GluK1 or GluK2 subunit partially reduced postsynaptic KA EPSCs, and GluK2 KO mice with a selective GluK1 antagonist UBP 302 could significantly reduce the KA EPSCs. These data suggest that both GluK1 and GluK2 play functional roles in the IC. Our study may provide the synaptic basis for the physiology and pathology of KA receptors in the IC-related functions.
**Introduction**

Cortical areas such as prefrontal cortex (PFC), the anterior cingulate cortex (ACC) and insular cortex (IC) are important for pain, emotion and memory process (Frankland et al. 2004; LaBar and Cabeza 2006; Zhuo 2008). Animal and human studies consistently suggest that neurons in the IC play important roles in pain and memory (Craig 2002; Jasmin et al. 2003; Shema et al. 2011). Electrophysiological recordings from in vivo animal found that insular cortical neurons respond to noxious gustatory, visceral and nociceptive stimuli (Benison et al. 2011; Gauriau and Bernard 2004; Hanamori et al. 1998). Human imaging data found that IC is activated by different pain conditions (Apkarian et al. 2005; Craig 2003; Eder et al. 2003; Henderson et al. 2007). In addition to sensory pain process, the IC has also been implicated in taste related learning and memory (Shema et al. 2011). Despite the important roles of the IC in sensory process and cognition, few studies are reported about synaptic transmission in the IC.

Glutamate, the major excitatory neurotransmitter in the central nervous system, activates three different receptors that directly gate ion channels, namely receptors for alpha-amino-3-hydroxy-5-methyl isoxozole propionic acid (AMPA), N-methyl-D-aspartate (NMDA), and kainate (KA). The KA receptor family is composed of five different subunits, namely GluK1, GluK2, GluK3, GluK4 and GluK5, which can form a variety of homomeric and heteromeric receptors. Most of basal synaptic responses are mediated by AMPA receptors, while NMDA and KA receptors are activated by repetitive synaptic stimulation (Hollmann and Heinemann 1994; Jane et al. 2009; Lerma 2003). However, in certain sensory and central synapses, KA receptors have also been reported to contribute to synaptic transmission. In the spinal cord dorsal horn, Li et al. (1999) reported that KA receptors contributes to sensory synaptic transmission (Li et al. 1999) activated by high-threshold nociceptive fibers. In 'Off' bipolar cells of mammalian retina, KA receptors mediate synaptic transmission from cones (DeVries and Schwartz 1999). In the ACC, Wu et al. (2005) reported that both GluK1 and GluK2 receptors contribute to KA transmission in layer II/III pyramidal neurons of the adult mice ACC (Wu et al. 2005) (see Table I). Although KA receptor mediated currents are much smaller than that of AMPA receptor, behavioral studies using gene knockout mice or pharmacological inhibitors show that different types of KA receptors participate in distinct physiological functions and pathological conditions (Jane et al. 2009; Ko et al. 2005; Wu et al. 2007). For example, selective and potent GluK1 antagonists inhibit formalin-induced paw licking, carrageenan-induced thermal hyperalgesia, and capsaicin-induced mechanical hyperalgesia (Dominguez et al. 2005; Jones et al. 2006). In gene knockout mice lacking GluK1, behavioral responses to capsaicin or formalin injection was significantly reduced (Ko et al. 2005). In addition, the mice lacking GluK2 show a significant impairment in fear memory (Ko et al. 2005).

These findings suggest possible contribution of KA receptors to sensory and memory-related brain areas such as the IC. In the present study, we employed integrative methods including whole-cell patch clamp recording, pharmacology and gene knockout mice. We characterized the function of KA receptors in glutamatergic synaptic transmissions. Electrophysiological mappings showed the KA functions in layer I, II/III and V/VI. Additionally, genetically modified mice with deletions of GluK1 or GluK2 were used to show that both GluK1 and GluK2 are involved in synaptic transmission in the IC.
KA receptor-mediated synaptic transmission in adult IC mouse neurons

Whole cell patch-clamp recordings were performed from visually identified pyramidal cells in layers II and III of IC slices in adult wild type (WT) mice, and a bipolar stimulation electrode was placed in layer V/VI of IC (n = 23 mice, Fig. 1A). The stimulation in the deeper layers may activate passing fibers from thalamo-cingulate pathway as well as local fibers (Lee et al. 2007). We recorded the neurons which receive pure monosynaptic synaptic input. We tested monosynaptic responses by delivering 20 shocks at 20 Hz (holding at -60 mV; Fig. 1B). Neurons with monosynaptic inputs followed the repetitive stimulation without failure. To test if excitatory synaptic transmission is mediated by glutamate, we bath applied an AMPA/KA receptor antagonist CNQX (20 μM). The evoked EPSCs were completely blocked by bath application of CNQX (20 μM) (n = 6 neurons/6 mice; Fig. 1C), indicating that glutamate is the major excitatory transmission in the IC.

Next, we examined if KA receptors contribute to synaptic responses in the IC neurons of adult mice (Fig. 2A). In the presence of a GABA<sub>A</sub> antagonist, picrotoxin (100 μM) and the selective NMDA receptor antagonist, AP-5 (50 μM), the evoked EPSCs by a single-pulse stimulation could be observed (n = 14 neurons/10 mice). After the baseline of EPSCs was stable recorded for at least 5 minutes, the selective and potent AMPA receptor antagonist GYKI 53655 (100 μM) was then applied through the bath solution to reveal KA mediated EPSCs. Finally, CNQX (20 μM) was applied and it blocked residual currents (Fig. 2A). As shown in Fig. 2B, GYKI 53655 had a fairly rapid and rigorous inhibitory effect on EPSCs in adult mice. A small residual EPSC persisted in the presence of GYKI 53655 10 min after perfusion. Perfusion of CNQX entirely blocked the residual GYKI 53655-resistant current (Fig. 2B), suggesting that the current was mediated by KA receptors. The pooled data of KA EPSCs was shown in Fig. 2D. The averaged components of KA EPSCs was 10.9 ± 2.0 % of the averaged AMPA/KA EPSCs as a baseline (averaged AMPA/KA EPSCs: 149.9 ± 7.7 pA; averaged KA EPSCs: 16.2 ± 3.2 pA, n = 14 neurons/10 mice). We also confirmed that an AMPA/KA receptor antagonist CNQX can block the KA EPSCs (2.1 ± 0.8 %, n = 5 neurons/5 mice, Fig. 2). Another AMPA receptor antagonist, SYM 2206 (100 μM) was also used to dissect the KA EPSCs in mice (Wu et al. 2005). Residual currents were observed 10 min after SYM 2206 perfusion. The averaged KA EPSCs of AMPA/KA EPSCs was 21.8 ± 4.2% (n = 4 neurons/4 mice). The discrepancy between the results obtained from GYKI 53655 and SYM 2206 may result from differences in the selectivity of the compounds (Wu et al. 2005). To test if GYKI 53655 at a higher dose may cause more inhibition, we applied 200 μM of GYKI 53655 for 10 min after 10 min GYKI (100 μM) treatment in same neurons. 200 μM GYKI 53655 did not cause any further inhibition (n = 5 neurons/3 mice, Fig. 2E).

KA receptor-mediated synaptic transmission in adult IC rat neurons

We further recorded pyramidal cells in layers II and III of adult rats (n = 5 rats, Fig. 2A and C). As followed mice recordings in Fig. 2A and B, we examined if KA receptors contribute to synaptic responses in the IC neurons of adult rats (Fig. 2). As shown in Fig. 2C, GYKI 53655 had a fairly rapid and rigorous inhibitory effect on EPSCs in adult rats. A small residual EPSC persisted in the presence of GYKI 53655 10 min after perfusion. Perfusion of CNQX entirely blocked the residual GYKI 53655-resistant current (Fig. 2C), suggesting that the currents in adult rats was also mediated by KA receptors. The pooled data of KA EPSCs is shown in Fig. 2D. In rats the summarized KA EPSCs was 7.0 ± 1.4%, of the baseline (averaged AMPA/KA EPSCs: 117.7 ± 20.0 pA; KA EPSCs: 7.2 ± 0.5 pA, n = 5 neurons/5 rats). As compared with KA currents in adult mice, there was not a statistically significant
difference between them (*p > 0.05). These results suggested that a relatively small component of fast excitatory synaptic transmission is mediated by synaptic KA receptors in the adult rodent IC.

**Slow kinetics and voltage dependence of KA receptor-mediated EPSCs**

Despite the rapid desensitization and deactivation of heterologously expressed KA receptors (Paternain 1998; Swanson and Heinemann 1998), most studies report that KA receptor-mediated EPSCs have slow kinetics (Ali 2003; Bureau et al. 2000; Cossart et al. 1998; DeVries and Schwartz 1999; Frerking et al. 1998; Kidd and Isaac 1999; Li et al. 1999; Wu et al. 2005). We performed a quantitative study of the kinetics of KA EPSCs in adult mice IC slices. As shown in Fig. 3A, KA EPSCs displayed slower kinetics than AMPA EPSCs. Both the decay time (13.5 ± 1.5 ms in AMPA vs. 85.2 ± 8.2 ms in KA, n = 14, *p < 0.01) and the rise time (10-90%) (3.8 ± 0.4 ms in AMPA vs. 10.0 ± 2.3 ms in KA, n = 14, *p < 0.05) of KA EPSCs were significantly slower than those of the AMPA EPSCs (Fig. 3A-C). The slow kinetics of KA EPSCs in IC pyramidal neurons are similar to those reported in many brain area, including spinal dorsal horn neurons (Li et al. 1999), hippocampal CA3 neurons (Castillo et al. 1997), thalamocortical neurons (Kidd and Isaac 1999) and the anterior cingulate cortex neurons (Wu et al. 2005).

To further characterize synaptic KA receptors, we studied the current-voltage (I-V) relationship of the KA EPSCs. The I-V relationship of KA receptors can reflect the calcium permeability and the subunit composition of channels (Egebjerg and Heinemann 1993; Ruano et al. 1995). In the presence of GYKI 53655, KA EPSCs were induced by single electric shocks. When recorded at various holding potentials ranging from -60 to +40 mV, KA EPSCs reversed at a potential of -0.15 ± 0.82 mV (n = 5 neurons/5 mice, Fig. 3D-E). The current recorded at the peak amplitude in relation to the holding potential was then plotted. Figure 3E illustrates the I-V curve of KA EPSCs in adult mice IC neurons, which displays a strong outward rectification (Fig. 3D and E). The mean rectification index of the KA EPSCs (ratio of estimated conductance at +40 and -60 mV) was 2.4 ± 0.8 (n = 5 neurons/5 mice). This finding suggests that the KA receptors involved in this response are likely of the edited, calcium impermeable form, as unedited receptors show strong inward rectification (Burnashev et al. 1996; Seeburg 1996).

**Summation properties of KA receptor-mediated EPSCs during repetitive stimulation**

In most synapses, brief repetitive impulse trains greatly facilitate KA receptor-mediated EPSCs (Castillo et al. 1997; Mulle et al. 1998; Vignes et al. 1997; Wu et al. 2005). To determine the summation properties of KA receptor-mediated synaptic responses in the mice IC, repetitive stimulations were applied by single, 5, 10 and 20 shocks at 200 Hz. As shown in Fig. 4A, in the presence of GYKI 53655, a small residual KA EPSCs was significantly increased in amplitude after repetitive stimulation (24.8 ± 4.1 pA by 5 shocks, 27.8 ± 3.8 pA by 10 shocks and 26.6 ± 3.8 pA by 20 shocks compared to single stimulations 12.6 ± 2.4 pA, n = 15 neurons/12 mice, *p < 0.05, see Fig. 4). 10 and 20 shocks at 200 Hz train significantly increased KA EPSCs compared with that induced by single shocks. No additional increase was observed by 20 shocks when compared with those obtained by 10 shocks, suggesting the saturation of the KA EPSCs (Fig. 4A and B).

**KA receptor-mediated EPSCs in wild-type, GluK1 KO or GluK2 KO mice**

Next we determined the subunit composition of synaptic KA receptors in adult mice IC neurons using mutant mice lacking GluK1 and GluK2 subunits. We employed two methods to evaluate the contribution of GluK1 and GluK2 in mediating the postsynaptic currents. First, we detected KA
receptor-mediated currents induced by single stimulation in GluK1 KO, GluK2 KO mice and GluK2 KO mice with bath applied a selective GluK1 antagonist, UBP 302 (10 μM) (Fig. 5A-C). As a result, GluK1 or GluK2 KO mice partially reduced KA EPSCs (6.2 ± 1.3%, n = 8 neurons/6 GluK1 KO mice; 8.2 ± 1.5%, n = 9 neurons/7 GluK2 KO mice). Interestingly, KA EPSCs were significantly blocked in GluK2 KO mice with bath applied UBP 302 (4.1 ± 0.4%, n = 7 neurons/7 GluK2 KO mice, compared with WT group, *p < 0.05, Fig. 5D). These results suggest that both GluK1 and GluK2 receptors may play the functional roles in the IC.

We further analyzed the summated KA receptor-mediated currents induced by high-frequency stimulations (single, 5, 10 and 20 shocks at 200 Hz) in GluK1 and GluK2 KO mice (Fig. 6A-B). High-frequency stimulations enhanced KA receptor-mediated currents in slices of wild-type, GluK1, GluK2 KO and GluK2 KO mice with UBP 302. As shown in Fig. 6A and B, the summations of KA EPSCs induced by repetitive stimulations in GluK1 or GluK2 KO mice were partially reduced compared with that of wild-type mice although there was not a significant difference in KA EPSCs between GluK1 and GluK2 KO mice (14.5 ± 2.1 pA by 5 shocks, 16.4 ± 2.8 pA by 10 shocks and 16.7 ± 4.4 pA by 20 shocks, n = 6 neurons/6 GluK1 KO mice; 14.7 ± 3.1 pA by 5 shocks, 16.0 ± 3.9 pA by 10 shocks and 17.6 ± 4.2 pA by 20 shocks, n = 7 neurons/6 GluK2 mice, p > 0.05). On the other hand, in GluK2 KO mice with UBP 302 group, a dramatic decrease in KA receptor mediated currents was also observed (6.6 ± 1.5 pA by 5 shocks, 6.9 ± 1.0 pA by 10 shocks, 6.4 ± 0.6 pA by 20 shocks, n = 6 neurons/6 GluK2 KO mice with UBP 302, *p < 0.05 compared with WT group), suggesting that both GluK1 and GluK2 receptors mediate the KA EPSCs. To further confirm the results, we compared the input (stimulation intensity)-output (KA EPSC amplitude) relationship of KA EPSCs in wild-type and KO mice. As shown in Fig. 6C, both GluK1 and GluK2 KO mice showed slightly decreased KA EPSCs (*p > 0.05, n = 6 neurons/6 GluK1 KO, n = 6 neurons/6 GluK2 KO). Furthermore, GluK2 KO mice with UBP 302 significantly decreased KA EPSCs compared with WT (n = 10 neurons/10 WT mice, n = 6 neurons/6 GluK2 KO mice with UBP 302, *p < 0.05). Taken together, these results indicate that both GluK1 and GluK2 underlie the synaptic KA receptor-mediated current in the IC.

**Comparison of KA receptor-mediated EPSCs in layer I, II/III and V/VI in mice IC neurons**

To further study KA receptor function in other layers of the IC, we next recorded neurons in layer I and V/VI (Fig. 7). By applying local stimulation in the layer II/III of the IC, we observed fast EPSCs from layer I neurons. Bath application of GYKI 53655 (100 μM) significantly reduced EPSCs, and small KA receptor-mediated currents were found in all neurons (7.7 ± 2.2%, n = 7 neurons, Fig. 7A and C). Subsequent application of CNQX (20 μM) completely blocked the responses, confirming that glutamate mediates fast synaptic responses.

We also recorded deep layer insular neurons (layer V/VI) by applying local stimulation in the layer II/III. We also found similar KA receptor-mediated currents as those in layer II/III neurons (9.7 ± 1.1%, n = 8, Fig. 7B and D). We compared pooled data of KA receptor-mediated currents in layer I, II/III and V/VI and the summarized data are shown in Fig. 7E.

To determine the summation properties of KA receptor-mediated synaptic responses in the IC, repetitive stimulations (single, 5, 10 and 20 shocks at 200 Hz) was applied from layer I and layer V/VI, respectively (10.8 ± 3.7 by 5 shocks, 11.4 ± 3.5 by 10 shocks, 11.1 ± 3.7 pA by 20 shocks, n = 6 layer I neurons/6 WT mice; 16.4 ± 2.3 by 5 shocks, 16.4 ± 3.0 pA by 10 shocks, 14.8 ± 2.4 pA by 20 shocks, n = 7 layer V/VI neurons/6 WT mice, p > 0.05, Fig. 8A-C). Furthermore, we compared the input-output
relationship of KA EPSCs in layer I and V/VI neurons. As shown in Fig. 8D, both layer I and V/VI neurons gradually increased the KA EPSCs along with stimulation intensity (n = 6 layer I neurons/6 mice and n = 7 layer V/VI neurons/6 mice). These results suggest that KA receptors play important roles in excitatory synaptic transmission within all layers of the IC.

Discussion

To our knowledge, the current work represents the first demonstration that KA subtype receptors contribute to fast excitatory synaptic transmission in IC neurons of adult rodents. Using whole cell patch-clamp recordings from IC neurons of brain slices, functional KA receptors were studied by single or train stimulation-evoked EPSCs. We mainly recorded neurons in layer II/III as well as in layer I and V/VI to detect electrophysiological mapping of postsynaptic KA functions in the IC. IC neurons in all layers have the functional KA receptors. Furthermore, using GluK1 or GluK2 KO mice, we provide the first evidence that both GluK1 and GluK2 contribute to functional synaptic KA receptors in layer II/III neurons. Considering the cumulative physiological evidence for the role of the IC in pain and higher brain functions, the present study provides important synaptic mechanisms for KA receptors in the IC related physiological and pathological functions.

KA mediated current in insular cortex

In the present study, the contribution of KA EPSCs evoked by single stimulations was very small in adult mice IC neurons (about 5-10% of AMPA/KA mediated currents in all layers of the mice IC and layer II/III in rats). The contribution of KA EPSCs in adult mice was similar in adult rats (Fig. 2). Moreover, saturated KA mediated EPSCs were nearly 30 pA after high-frequency repetitive stimulations (Fig. 4). A small percentage of KA EPSCs compared with AMPA/KA EPSCs is reported in many preparations; for example, Golgi cells in the cerebellum (Bureau et al. 2000), layer V pyramidal neurons in the neocortex (Eder et al. 2003) and the anterior cingulate cortex (Wu et al. 2005). However, a comparatively bigger component of KA receptor-mediated EPSCs was present in thalamocortical synapses (Kidd and Isaac 1999), spinal dorsal neurons (Li et al. 1999) and the basolateral amygdala (Li et al. 2001; Li and Rogawski 1998). Possible explanations are that calcium permeability of GluK receptors in the IC may be different from other areas (Burnashev et al. 1996; Seeburg 1996). RNA editing generates different forms for KA receptor subunits GluK1 and GluK2. These subunits carry alternative residues in the Q/R site of their M2 region (Sommer et al. 1991). The Q/R site in GluK1 and GluK2 is important for calcium permeability of the glutamate activated channel (Egebjerg and Heinemann 1993; Köhler et al. 1993). Indeed, the strong outward rectification of the I-V curve for KA EPSC was observed in adult IC neurons (Fig. 3). Similar results were reported in pyramidal neurons in layer II/III and layer V in motor cortex (Ali 2003), and in layer II/III in the ACC (Wu et al. 2005). The use of adult mice in the present study may partially explain the small amplitude of KA EPSCs. The Q/R site in GluK1 and GluK2 is subject to developmentally controlled pre-mRNA editing (Seeburg 1996). For example, GluK1 transcripts are edited by shifting 6-50% during embryonal day 15 to adult rats in hippocampus. For GluK2, 30-70% of Q/R site in GluK2 KO is edited in the embryonal day 15 to adult in hippocampus. KA receptors at neocortical synapses during this developmental period also exhibit a strongly inward rectifying I-V relationship (Kidd and Isaac 1999), suggesting they contain a substantial proportion of unedited subunits.

KA receptor mediated EPSC kinetics

In adult IC slices, we observed slow kinetics of KA EPSCs compared with AMPA EPSCs in the same cells. This result is similar as other central nerves systems (Ali 2003; Bureau et al. 2000; Cossart et
al. 1998; DeVries and Schwartz 1999; Frerking et al. 1998; Kidd and Isaac 1999; Li et al. 1999; Wu et al. 2005). It is generally accepted that the kinetics of KA EPSCs might be due to intrinsic properties of these postsynaptic receptors (Lerma 2003). What other mechanisms might explain the slow kinetics?

One possibility is that KA receptors interact with proteins at the synapse and this causes a change in their kinetic properties (Bowie et al. 2003; Garcia et al. 1998; Straub et al. 2011a; Straub et al. 2011b; Zhang et al. 2009). Recently Neuropilin and Tolloid like (Neto) family were identified as a novel accessory subunit of KARs (Zhang et al. 2009). The KAR auxiliary subunit Neto1 regulate the slow kinetics in hippocampus (Straub et al. 2011a). Neto2 interacts specifically with recombinant and native GluK1 and GluK2 and modulates KAR currents in vitro and in vivo (Straub et al. 2011b). Furthermore, a study has provided evidence for this by showing that interactions of GluK2 homomers or GluK2/GluK5 heteromers with the postsynaptic density protein SAP 90 produced receptors that did not fully desensitize in response to glutamate (Bowie et al. 2003; Garcia et al. 1998).

However, KA EPSCs evoked by single, repetitive stimulations at 200 Hz and input-output relationship were not completely blocked by genetic deletions of GluK2 receptor combined with the application of GluK1 antagonist. We consider that the residual components of KA EPSCs may be mediated by other KA receptors subunits or heteromeric KA receptors since GluK1-5 receptors mRNA subunit widely expressed in many cortical regions and hippocampus (Bahn et al. 1994). KA receptors are known to be composed of both homomeric and heteromeric co-assemblies such as GluK1, GluK2, GluK3, GluK1/GluK5, GluK2/GluK5 and GluK1/GluK2 (Hollmann and Heinemann 1994; Jane et al. 2009; Lerma et al. 2001). It still remains unclear the co-assemblies with GluK1 and GluK2 or GluK1, GluK2 combined with other subunits in the IC. To dissect the exact composition of KA receptors in the IC, selective KO mice for the other KA receptor subunits as well as selective pharmacological tools are needed in future studies. Another possibility is that synaptic KA receptors are heteromers that have novel properties and that these combinations of subunits are not found for the native extrasynaptic or heterologously expressed KA receptors so far studied. The properties of GluK2 homomers are well known; they exhibit rapid kinetics similar to AMPA receptors (Herb et al. 1992; Swanson 1996; Swanson and Heinemann 1998). While there is no information on GluK1(Q)/GluK3/GluK5 heteromers, it is known that GluK1(Q)/GluK5 also exhibits rapid kinetics (Herb et al. 1992). However, co-assembly of different subunits can cause alterations in the properties of the receptor complex (Cui and Mayer 1999; Herb et al. 1992), and GluK1- containing receptors exhibit variations in kinetics, for example, in the rate of recovery from desensitization (Swanson and Heinemann 1998). These properties could provide potential mechanisms for the slow kinetics of synaptic KA receptors.

Functional roles of IC and possible involvement of KA receptors

Recent studies from both humans and animals suggest that the IC is important for the processing of sensory information, memory, emotion, and other higher-order brain functions (Calejesan et al. 2000; Casey et al. 1996; Craig 2009; Davis et al. 2000; Davis et al. 1997; Devinsky et al. 1995; Donahue et al. 2001; Eisenberger et al. 2003). Previous results show that the IC is involved in pain and fear memory in rodents (Shema et al. 2011; Wei et al. 2002; Wei et al. 2001). In addition, previous anatomical study shows that the IC receives input from the thalamus, amygdala, hippocampus and the ACC and communicates with these brain nucleus (Jasmin et al. 2004). It is well known that the amygdala, hippocampus, as well as ACC play important roles in pain and fear memory (Johansen et al. 2011; Zhuo 2008). Our previous studies have indicated that different subunits of KA receptors show distinct behavioral phenotypes in pain and fear memory (Ko et al. 2005). GluK2 KO mice show a significant impairment in synaptic plasticity within the amygdala and auditory cortex, and deficit in fear memory.
test (Ko et al. 2005). On the other hands, mice lacking GluK1 significantly reduce the responses to capsaicin or inflammatory pain (Ko et al. 2005). Combined with these evidences and this study, the functional GluK1 and GluK2 receptors in the IC may underlie the synaptic basis for IC-related brain functions, such as pain and memory.

Methods

Animals

Adult C57BL/6 mice and Sprague-Dawley rats were purchased from Charles River (8-14 week old). GluK1 and GluK2 KO mice were obtained as gifts from Stephen F. Heinemann (Salk Institute, San Diego, CA) (Mulle et al. 1998; Sailer et al. 1999). For experiments using KO mice, GluK1 and GluK2 were maintained on a mixed 129Sv X C57BL/6 background and wild-type littermates were used as controls. The GluK1 and 2 KO mice were generated by crossing homozygous GluK1 and GluK2 KO mice. All mice were maintained on a 12-h light/dark cycle with food and water provided ad libitum. The Animal Care and Use Committee at the University of Toronto approved the experimental protocols. All experiments related to mutant mice were performed blind to the genotype.

Whole cell patch-clamp recordings in adult IC slices

Rodents were anesthetized with 1-2% isoflurane. Transverse brain slices of the IC (300 μm) were prepared using standard methods (Li et al. 2010; Wei et al. 2002; Wei et al. 2001; Wu et al. 2005). Slices were transferred to a room temperature-submerged recovery chamber with an oxygenated (95% O₂-5% CO₂) solution containing (in mM) 124 NaCl, 25 NaHCO₃, 2.5 KCl, 1 KH₂PO₄, 2 CaCl₂, 2 MgSO₄ and 10 glucose. After a 1-h recovery period, slices were transferred into a recording chamber on the stage of an Axioskop 2FS microscope (Carl Zeiss) equipped with infrared DIC optics for visualizing whole cell patch-clamp recordings. Excitatory postsynaptic currents (EPSCs) were recorded from layer II/III and V/VI pyramidal neurons and layer I neurons with an Axon 200B amplifier (Axon Instruments) in the IC and stimulation was delivered by a bipolar tungsten-stimulating electrode placed in layer V/VI of the IC for layer II/III recording, in layer II/III for layer V/VI and layer I recording, respectively (Fig. 1A & Fig. 7A). Control test pulses were given every 30 s. For frequency facilitation, repetitive stimulation was delivered at 200 Hz (5, 10, or 20 shocks). In the voltage-clamp configuration, recording electrodes (2-5 MΩ) contained the pipette solution composed of (in mM) 120 Cs-gluconate, 5 NaCl, 1 MgCl₂ 0.5 EGTA, 2 Mg-ATP, 0.1 Na₃GTP, 10 HEPES, and 2 lidocaine N-methyl bromide quaternary salt (QX-314), pH 7.2; 280-300 mOsm. The initial access resistance was 15-30 MΩ, and it was monitored throughout the experiment. Data were discarded if the access resistance changed > 15% during experiment. Data were filtered at 1 kHz, and digitized at 10 kHz. The membrane potential was held at -60 mV throughout the experiment.

Chemicals and drug application

All chemicals and drugs were obtained from Tocris (St. Louis, MO). All experiments were conducted in the presence of picrotoxin (PTX; 100 μM) and D-2-amino-5-phosphono-pentanoic acid (AP-5; 50 μM).

Data analysis

Data are presented as means ± SEM. Statistical comparisons between two groups were performed using two-tail paired or unpaired t-test, and between more than three groups were performed using
Dunnett test to identify significant differences. In layers II/III, I, V/VI of WT, GluK1 KO, GluK2 KO and GluK2 KO mice with UBP 302, the recordings by repetitive stimulations and input-output were analyzed by both analysis of variance between groups (one-way repeated ANOVA) (within each group) and two-way repeated ANOVA (Student-Newmann-Keuls test to compare groups). In all cases, \( *p < 0.05 \) was considered statistically significant. Time constants for EPSCs were obtained by fitting one exponential function to the falling phase of the currents.

**List of Abbreviations**

ACC; Anterior cingulate cortex  
AMPA; \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid  
AP-5; D-2-amino-5-phosphono-pentanoic acid  
CNQX; 6-cyano-7-nitroquinoxaline-2, 3-dione  
EPSCs; Excitatory postsynaptic currents  
IC; Insular cortex  
KA; Kainate  
NMDA; N-methyl-D-aspartate  
PTX; picrotoxin  
WT; wild type

**Competing interests**

The authors declare that they have no competing interests.

**Authors' contributions**

KK, SS, TC and LJW performed electrophysiology and drafted the manuscript. MZ and BKK designed and finished the final draft of the manuscript. All authors read and approved the final manuscript.

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Legend

Figure 1 Recordings of monosynaptic EPSCs from layer II/III in IC
(A) Recording diagram of layer II/III pyramidal neurons and stimulation in layer V/VI of adult mice IC.
(B) Monosynaptic input by 20 shocks at 20 Hz.
(C) AMPA/KA mediated EPSCs were recorded in the presence of picrotoxin (PTX, 100 μM) and D-2-amino-5-phosphono-pentanoic acid (AP-5, 50 μM) (baseline, a). The evoked EPSCs were completely blocked by a AMPA/KA antagonist, 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX, 20 μM) (b). In the following example of EPSCs, each trace represents an average of 5-10 consecutive recordings.

Figure 2 KA receptor-mediated EPSCs in pyramidal neurons of layer II/III IC in adult mice and rats
(A) Experimental procedure of detecting KA receptor-mediated EPSCs by pharmacological tools, an AMPA antagonist, GYKI 53655 (100 μM) and CNQX (20 μM).
(B-C) In mice (B) and rats (C), AMPA/KA EPSCs were recorded in the presence of PTX (100 μM) and AP-5 (50 μM) for 5 min (a). After the perfusion of GYKI 53655 (100 μM) for 10 min, a small residual current remained (b) and they could be totally blocked by CNQX (20 μM) (c). Sample points showing the time course of GYKI 53655 and CNQX perfusion on the EPSCs shown in B and C (d).
(D) Statistical results showing the percentage of EPSCs in the presence of (±)-4-(4-aminophenyl)-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7-methylenedioxyphthalazine (SYM 2206) (n = 4 from 3 mice), GYKI 53655 (n = 9 from 5 mice, n = 5 from 4 rats), and CNQX (n = 5 from 4 mice). *p < 0.05 shows the statistical difference between the amplitudes of baseline and results of pharmacological drugs.
(E) The comparison of GYKI 53655 (100 μM) with a higher dose, 200 μM in the same neurons (n = 5 from 3 mice).

Figure 3 KA receptor-mediated EPSCs show slower kinetics and rectified I-V curve
(A) Superimposed traces showing control EPSCs, EPSCs after application of GYKI 53655, or CNQX.
(B) Normalized traces of GYKI sensitive and resistant EPSCs.
(C) Pooled data of rise time and decay time in GYKI-sensitive and -resistant traces.
(D) Representative KA EPSCs at holding potentials from -60mV to +40 mV.
(E) I-V curve of KA EPSCs plotted from current amplitude vs. various holding potentials.

Figure 4 High frequency stimulation-dependent summations of KA receptor-mediated EPSCs of layer II/III neurons
(A) Representative traces of KA EPSCs obtained after different number of stimulations at a frequency (single, 5, 10 and 20 shocks) of 200 Hz (a-d).
(B) Layer II/III neurons show a strong increase by repetitive stimulations (single, 5, 10 and 20 shocks)
(One-way repeated ANOVA, n = 15 from 12 mice, p < 0.05). Note that 10 shocks induced a saturated current.

Figure 5 KA-mediated EPSCs in WT, GluK1, GluK2 and GluK2 KO mice with a selective GluK1 antagonist
(A-C) KA-mediated EPSCs in GluK1 (A), GluK2 (B) and GluK2 KO mice with UBP 302 (10 μM), a selective GluK1 antagonist (C).
(D) Pooled data of KA-EPSCs in WT (n = 14), GluK1 (n = 8), GluK2 (n = 9) and GluK1 KO mice with
UBP 302 (n= 7). The averaged amplitude of KA-mediated currents in WT and GluK2 KO with UBP 302 were significantly different (Dunnett test, *p < 0.05).

**Figure 6** Comparisons of repetitive stimulation-induced KA EPSCs in WT, GluK1 KO, GluK2 KO and GluK2 KO mice with UBP 302
(A) Repetitive stimulation (10 shocks at 200 Hz) induced KA mediated EPSCs in WT, GluK1, GluK2 KO and GluK2 KO mice with UBP 302.
(B) Pooled data of repetitive stimulations (single, 5, 10 and 20 shocks) at 200 Hz in WT (n = 15 from 10 mice), GluK1 (n = 6 from 6 mice), GluK2 KO (n = 7 from 6 mice) and GluK2 KO mice with UBP 302 (n = 6 from 6 mice). While WT and GluK2 KO groups significantly increased the summation amplitudes by repetitive stimulations (Fig. 4B), GluK1 and GluK2 KO with UBP 302 groups show no such significant increase (One-way repeated ANOVA). Importantly, a strong difference was observed between WT and GluK2 KO mice with UBP 302 groups (Two-way repeated ANOVA, *p< 0.05).
(C) Input-output relationship for single-shock-induced KA receptor EPSCs. While WT mice increased the amplitudes of KA-mediated EPSCs stimulation intensity-dependently (One-way repeated ANOVA, n = 14 from 10 mice, *p< 0.05), GluK1 (n = 6 from 6 mice), GluK2 KO (n = 7 from 6 mice) and GluK2 KO mice with UBP 302 (n = 6 from 6 mice) groups did not show significant increase. Note that a significant difference was observed between WT and GluK2 KO with UBP 302 groups (Two-way repeated ANOVA, *p< 0.05).

**Figure 7** Recordings of KA EPSCs from layer I, V/VI and II/III in IC
(A) Recording diagram of layer I neurons and stimulation in layer II/III of adult mice IC.
(B) Recording diagram of layer V/VI pyramidal neurons and stimulation in layer II/III of adult mice IC.
(C-D) In layer I (C) and V/VI (D), AMPA/KA EPSCs were recorded in the presence of PTX (100 μM) and AP-5 (50 μM) (baseline in a). After the perfusion of GYKI 53655 (100 μM), a small residual current remained (b) and they could be totally blocked by CNQX (20 μM). Sample points showing the time course of GYKI 53655 and CNQX effects on the neuron shown in C and D.
(E) Pooled data of KA EPSCs in layer I (n = 7), V/VI (n = 8) and II/III neurons (n = 15).

**Figure 8** Comparisons of KA receptor-mediated EPSCs in layer I, II/III and V/VI
(A-B) Representative traces of KA EPSCs obtained after different number of stimulation at a frequency (single, 5, 10 and 20 shocks) of 200 Hz (a-d) in layer I (A) and V/VI neurons (B).
(C) Pooled data of repetitive stimulations (single, 5, 10 and 20 shocks) by 200 Hz in layer I (n = 6), V/VI (n = 7) and II/III (n = 15). While layer II/III neurons significantly increased the summation amplitudes by repetitive stimulations (Fig. 4B), layer I and V/VI neurons show no such significant increase (One-way repeated ANOVA). Importantly, a strong difference was observed between layer II/III and I KO neurons (Two-way repeated ANOVA, *p< 0.05).
(D) Input-output relationship for single-shock-induced KA receptor EPSCs. Intensity-dependent summation of KA receptor-mediated EPSCs in layer I (n = 6), V/VI (n = 7) and II/III (n = 15). While layer II/III neurons increased the amplitudes of KA-mediated EPSCs stimulation intensity-dependently (One-way repeated ANOVA, n = 15 from 10 mice, *p< 0.05), layer I and V/VI (n = 7) neurons showed no such significant increase.
Table I. KA receptors in mammalian central synaptic transmission and regulation

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Swanson PH. The role of RNA editing in controlling glutamate receptor channel properties. *J Neurochem* 66: 1-5, 1996.


Figure 1

A

Patch-clamp recording in layer III of the mouse IC

B

Monosynaptic EPSCs

20 shocks at 20 Hz

C

AMPAR/KA EPSCs (baseline)  b  CNQX (20 μM)
Figure 2

A

5 min
Baseline
in PTX and AP-5

10 min
GYK 93655 (100 μM)
a AMPA receptor antagonist

CNQX (20 μM)
a AMPA/Kainate receptor antagonist

B

in layer III of mice IC

Baseline

GYK 93655

CNQX

d

GYK 93655

CNQX

d

EPSC amplitude (μA)

Time (min)

180
120
60
0

5
10
15
20
25

40 μA

30 ms

C

in layer III of rats IC

Baseline

GYK 93655

CNQX

d

GYK 93655

CNQX

d

EPSC amplitude (μA)

Time (min)

180
120
60
0

5
10
15
20
25

40 μA

30 ms

D

Relative amplitude (%)

0
10
20
30
40
50
60
70
80
90
100

Time (min)

0
5
10
15
20
25

E

Relative amplitude (%)

0
10
20
30
40
50
60
70
80
90
100

0
5
10
15
20
25

Over expression
Over expression + CNQX
Figure 3

A

B

C

D

E
Figure 4

A

a  Single shock

b  5 shocks at 200 Hz

c  10 shocks at 200 Hz
d  20 shocks at 200 Hz

B

- Graph showing EPSC amplitude (pA) vs. number of shocks at 200 Hz.

- Inset: EPSCs in layer 2/3 (n = 15).

- Scale bars: 10 pA, 80 ms.

- Y-axis: EPSC amplitude (pA)

- X-axis: Number of shocks at 200 Hz
Figure 5

A

GluK1 KO
Baseline

b
GYKI 53655

B

GluK2 KO
Baseline

b
GYKI 53655

C

GluK2 KO + UBP 302
Baseline

b
GYKI 53655

D

Relative amplitude (%)

A
Figure 6

A 10 shocks at 200 Hz

B

C

WT (n = 16)
GluK1 KO (n = 8)
GluK2 KO (n = 7)
GluK2 KO + UBP 382 (n = 8)

BPS amplitude (µA)
Number of shocks at 200 Hz

BPS amplitude (µA)
Stimulation intensity (V)
Figure 8

A. In layer I
   a. Single shock
   b. 5 shocks at 200 Hz
   c. 10 shocks at 200 Hz
   d. 20 shocks at 200 Hz

B. In layer V/Vi
   a. Single shock
   b. 5 shocks at 200 Hz
   c. 10 shocks at 200 Hz
   d. 20 shocks at 200 Hz

C. EPSCs amplitude (pA) vs. Number of shocks at 200 Hz

D. EPSC amplitude (pA) vs. Stimulation intensity (V)

- ▲: In layer I (n = 10)
- ▼: In layer V/Vi (n = 7)
- ●: In layer V/Vi (n = 10)